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Marine fungi from Sarushima Island, Japan, with a phylogenetic evaluation of the genus *Naufragella*

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ABSTRACT Twenty-seven fungi (18 ascomycetes, 9 anamorphic fungi) were recorded from 91 driftwood samples collected from Sarushima Island, Japan. *Ceriosporopsis halima*, *Corollospora maritima*, and *Halosphaeria appendiculata* occurred most frequently in the fungal community. Other common fungi include: *Remispora maritima*, *Tirisporea* sp., *Monotosporella* sp., *Phoma* sp., and *Trichocladium achrasporum*. Among the 27 fungi collected, 7 are possibly new to science. *Nemania maritima* and *Panorbis viscosus* are new records for Japan. Phylogenetic analyses of LSU rDNA sequences placed *Naufragella delmarensis* with *Nohea umiumi* in a highly supported clade. Both *Naufragella* and *Nohea* have similar morphological characters that, in combination with the molecular data, support their placement in a single genus. The new combinations *Nohea delmarensis* and *N. spinibarbata* are proposed. The anamorph teleomorph connection of *Trichocladium achrasporum* *Halosphaeriopsis mediosetigera* was supported with molecular data for the first time.

KEY WORDS — ecology, *Halosphaeriales*, sub-tropics, aquatic fungi

Introduction

Our knowledge of lignicolous marine fungi from coastal areas of Japan is fragmentary, with only a few studies carried out with wood blocks baits in oceanic water (Tubaki 1966, 1967, 1969, 1973). These four studies documented the presence of 25 lignicolous marine fungi, including a new species, *Remispora galerita* Tubaki (Tubaki 1967). Sarushima Island, the only natural island in Tokyo Bay, was a military fort until the Second World War when it was closed to civilian access. Sarushima Island is now a tourist site and the public has easy access to the island. As no marine fungi have been reported from this small island, this study was carried out to document its marine fungal diversity.

The genera *Nohea* and *Naufragella* are closely similar in their morphology. Both genera have two types of ascospore appendages: gelatinous and fibrous. The two genera differ only in the position of the ascospore appendages. *Nohea* has gelatinous appendages attached subapically and laterally and two lateral fascicles of thin hairs. *Naufragella* has apical gelatinous appendages and a subapical crown of filaments at each apex below the gelatinous appendages. Kohlmeyer and Volkmann–Kohlmeyer (1998) opined that the differences in the positions of the gelatinous appendages (lateral in *Nohea* vs. apical in *Naufragella*) and filaments (on one side in *Nohea* vs. forming a subapical crown in *Naufragella*) are distinctive characters that can be used to separate fungi at the generic level. Phylogenetic analyses herein were carried out to test whether the genera *Nohea* and *Naufragella* are congeneric. Another closely similar genus, *Remispora*, is characterized by its wing-like apical, gelatinous ascospore appendages that unfold to form long fibrous material in water.

During this study, phylogenetic analyses of LSU rDNA sequences were carried out to elucidate the relationships among the genera *Naufragella*, *Nohea*, and *Remispora* as well as among various marine species.

Materials and methods

Collection techniques

Samples of driftwood collected from the intertidal zone of Sarushima Island, Japan, were placed in clean plastic bags and returned to the laboratory. They were examined with a dissecting microscope for marine fungi immediately upon return to the laboratory, placed in sterile humid plastic boxes for incubation, and examined periodically over 4 months of incubation. Methods used for the preparation of materials for light microscopy followed those of Jones & Hyde (1988). For each taxon, percent occurrence was calculated as: $(\text{no. of collections}) \times 100 / (\text{no. of samples})$.

Digital micrographs were obtained with a Nikon Eclipse E800 differential interference contrast light microscope and “studio viewfinder version 3” (Nikon Corporation, Japan) digital imaging system. Wood samples bearing fungi were dried at 60 °C for 24 h. To obtain single-ascospore cultures of the recorded fungi, ascomata were cut open with a sterile razor blade, and the centrum tissue containing ascospores was removed with sterile forceps and placed in sterile seawater. Small drops of this ascospore suspension were placed on GYA (10 g glucose, 1 g yeast extract, 18 g agar in 1 l seawater) in Petri dishes and incubated at 25°C in the dark. Germinated ascospores were transferred to new GYA Petri dishes with sterile forceps and incubated at 25°C in the dark. Dried fungi and isolated fungal cultures were deposited at the Extremobiosphere Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Japan and National Institute of Technology and Evaluation, Biological Resource Center (NBRC), Japan. DNA isolation, PCR reactions, cycling parameters, sequencing, sequence alignment and phylogenetic analyses were carried out as described by Abdel-Wahab et al. (2009).

DNA extraction, sequencing, and phylogenetic analysis

DNA was extracted from pure fungal mycelium using the Microbial DNA Extraction Kit (MOBIO; Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Partial LSU ribosomal DNA was amplified using primers LR0R and LR7 (Bunyard et al. 1994). PCR reactions, cycling parameters and sequencing were carried out as described by Abdel-Wahab et al. (2009). Sequences were assembled using Sequencher 4.2.2 (Gene Codes Corporation). Sequences were aligned with others retrieved from GenBank using ClustalX (Thompson et al. 1997) and optimized manually. The positions where one or more species contained a length mutation and ambiguously aligned regions were not included in the subsequent phylogenetic analysis. Nucleotide sequence phylogenies were constructed using PAUP* 4.0b10 (Swofford 2002). Maximum likelihood (ML) analyses (Felsenstein 1981) were performed using heuristic searches with the random stepwise addition of 100 replicates and tree bisection reconnection (TBR) rearrangements. The optimal model of nucleotide substitution for the ML analyses was determined using hierarchical likelihood ratio tests as implemented in Modeltest 3.7 (Posada & Crandall 1998). The model selected as the best fit for LSU rDNA data set was TrN+I+G. For the bootstrap analyses (Felsenstein 1985), 100 replicates were generated with 5 random additions and TBR. Maximum parsimony (MP) trees were obtained by 100 random addition heuristic search replicates using PAUP, and 1000 bootstrap replicates were performed employing 5 random addition heuristic searches. Posteriori probability values were obtained by using the MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) with the GTR+I+G model that was determined using MrModeltest 2.2 (Nylander 2004). Nodal supports in the Bayesian analyses were examined by posterior probabilities from five million generations that were run in four chains with sampling every 100 generations, yielding 50 000 trees, of which the first 12 500 were discarded as "burn in." Five sequences of the LSU rDNA sequences were generated in this study and were deposited at GenBank and their accession numbers are shown in FIG. 1.

Results

Twenty-seven fungi (18 ascomycetes and 9 anamorphic fungi) were identified from 122 fungal collections that were recorded from 91 driftwood samples collected from Sarushima Island, Japan. *Ceriosporopsis halima* (17.6 %), *Corollospora maritima* (43.9 %) and *Halosphaeria appendiculata* (12.1 %) occurred most frequently. Other common fungi include: *Remispora maritima* (5.5 %), *Tirisporea* sp. (8.8 %), *Monotosporella* sp. (5.5 %), *Phoma* sp. (7.7 %) and *Trichocladium achrasporum* (5.5 %). Among the 27 fungi recorded during the present study, 7 fungi (representing 25.9 % of the recorded fungi) are possibly new to science. *Nemania maritima* and *Panorbis viscosus* are reported from Japan for the first time. Representatives of the *Halosphaeriales* predominated in the fungal community, comprising 27 species (59.3 %). Hyphomycetes, represented by 8 species, comprised the major anamorphic group, and coelomycetes were represented by one species of *Phoma* (TABLE 1).

TABLE 1. Frequency of occurrence of marine fungi collected from Sarushima Island

| FUNGI | % |
|---|------|
| Ascomycota | |
| <i>Botryosphaeria</i> sp. | 1.1 |
| <i>Ceriosporopsis halima</i> Linder | 17.6 |
| * <i>Ceriosporopsis</i> sp. 1 | 1.1 |
| * <i>Ceriosporopsis</i> sp. 2 (MF 985) | 3.3 |
| <i>Corollospora maritima</i> Werderm. | 43.9 |
| * <i>Dryosphaera</i> sp. | 1.1 |
| <i>Halosphaeria appendiculata</i> Linder | 12.1 |
| <i>Halosphaeriopsis mediosetigera</i> (Cribb & J.W. Cribb) T.W. Johnson | 2.2 |
| <i>Leptosphaeria</i> sp. | 1.1 |
| <i>Lulworthia grandispora</i> Meyers | 1.1 |
| <i>Marinospora calyptrata</i> (MF986) | 3.3 |
| * <i>Morakotiella</i> sp. (MF 980) | 2.2 |
| <i>Nemania maritima</i> Y.M. Ju & J.D. Rogers | 2.2 |
| <i>Panorbis viscosus</i> (I. Schmidt) J. Campb. et al. | 1.1 |
| <i>Nohea delmarensis</i> (MF982) | 1.1 |
| <i>Remispora maritima</i> (MF988) | 5.5 |
| * <i>Remispora</i> sp. (MF987) | 1.1 |
| * <i>Tirisporea</i> sp. (MF960) | 8.8 |
| Anamorphic fungi | |
| <i>Cirrenalia macrocephala</i> (Kohlm.) Meyers & R.T. Moore | 1.1 |
| <i>Halazoon fuscus</i> (I. Schmidt) Abdel-Wahab et al. | 2.2 |
| * <i>Monotosporella</i> sp. (MF984) | 5.5 |
| <i>Phoma</i> sp. | 7.7 |
| <i>Trichocladium achrasporum</i> | 5.5 |
| <i>Trichocladium melhae</i> E.B.G. Jones et al. | 1.1 |
| <i>Xylomyces</i> sp. | 1.1 |
| <i>Veronaea japonica</i> Arzanlou et al. (MF991) | 1.1 |
| Unknown synnematosus fungus | 1.1 |

*Possibly new fungi

Total numbers: samples 91; fungal collections 122; species 27; ascomycetes 18; anamorphic fungi 9; fungi per sample 1.3.

Phylogenetic analyses of *Halosphaeriales* LSU rDNA dataset

The partial LSU rDNA sequences of *Marinospora calyptrata*, *Naufragella delmarensis*, *R. maritima*, and *T. achrasporum* were aligned with representatives of the *Halosphaeriales* along with representatives of the *Microascales* and *Xylariales*. In total, the dataset included 36 taxa of which 31 belong to *Halosphaeriales*, one to *Microascales*, and four to *Xylariales* that were used as outgroup taxa. The dataset consisted of 460 total characters, of which 49 gaps were excluded, 250 characters were constant, 48 variable characters were parsimony-uninformative, and 113 were parsimony informative characters.

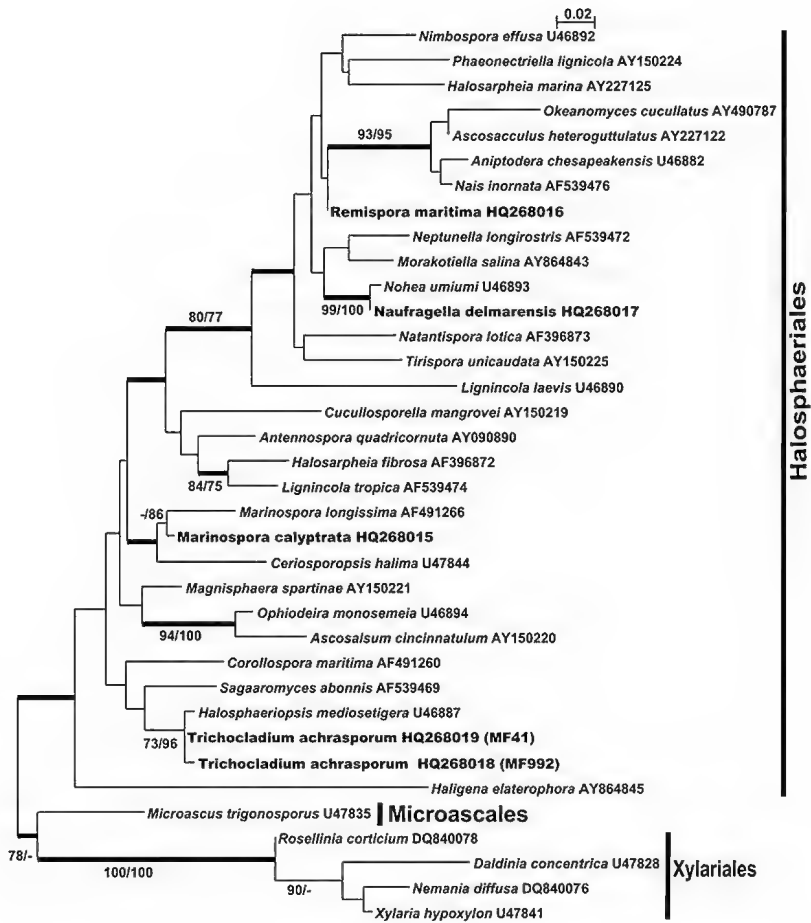


FIG. 1: Maximum likelihood phylogenetic tree ($-\ln$ likelihood = 3,008.01) shows relationships of *Marinospora calyptrata*, *Naufragella delmarensis*, *Remispora maritima*, and *Trichocladium achrasporum* and representatives of halosphaeriaceous ascomycetes based on LSU rDNA. The numbers indicate pp values > 95% (in bold), ML bootstrap and MP bootstrap values > 70%. Sequences generated during the present study are set in bold font.

The heuristic search generated 24 most parsimonious trees, all 520 steps long, and with a consistency index of 0.4462, a retention index of 0.6449, and a rescaled consistency index of 0.2877. Maximum likelihood analysis produced one tree with an $-\ln$ likelihood score of 3,008.01 (FIG. 1). Most parsimonious (MP) and Bayesian analyses produced trees (not shown) similar to the one in FIG. 1.

Taxonomic notes

Naufragella Kohlm. & Volkm.-Kohlm.

Kohlmeyer & Volkmann-Kohlmeyer (1998) established the genus *Naufragella* Kohlm. & Volkm.-Kohlm. to accommodate a new fungus, *N. delmarensis*, and also transferred *Remispora spinibarbata* to the new genus. During this study *N. delmarensis* was isolated, described, photographed (FIGS 2–5), and sequenced.

The genera *Naufragella* and *Remispora* Linder share common morphological characters. Barghoorn & Linder (1944) established the genus *Remispora* to accommodate halosphaeriaceous fungi with exosporic ascospore appendages that consist of a fibrous component in an amorphous matrix. Fragmentation of the sheath gives rise to wing-like fibrous polar appendages (Jones & Moss 1978, Johnson et al. 1984, Jones et al. 2009). Recent phylogenetic analyses revealed that *Remispora* is polyphyletic (Jones et al. 2009).

In contrast, *Naufragella* and *Nohea* both have two types of appendages: (a) gelatinous, forming a band or veil at each apex and (b) a crown of delicate filaments below each apex (Kohlmeyer & Volkmann-Kohlmeyer 1998). Phylogenetic analyses place the type species of both genera, *Nohea umiumi* and *Naufr. delmarensis*, within a highly supported clade (99/100/100 for ML/MP/Bayesian pp respectively) that forms a sister group to *Morakotiella salina* (C.A. Farrant & E.B.G. Jones) Sakay. and *Neptunella longirostris* (Cribb & J.W. Cribb) K.L. Pang & E.B.G. Jones. I conclude that the genera, *Nohea* and *Naufragella* are congeneric and that, within the *Halosphaeriales*, ascospore appendage position is not a reliable character with which to separate fungi at the generic level, contrary to the opinion of Kohlmeyer & Volkmann-Kohlmeyer (1998). *Naufragella spinibarbata* is very similar to *Naufr. delmarensis*, and the two species differ only in ascospore dimensions and appendage shape and length. *Naufragella spinibarbata* was not available for sequencing and further study is needed to confirm its phylogenetic relationships but for the time being it should returned to *Nohea*.

Based on the morphology and the molecular results presented here, the following two combinations are proposed:

***Nohea spinibarbata* (Jørg. Koch) Abdel-Wahab, comb. nov.**

MYCOBANK MB 519505

≡ *Remispora spinibarbata* Jørg. Koch, Nordic Journal of Botany 8: 517, 1989.

≡ *Naufragella spinibarbata* (Jørg. Koch) Kohlm. & Volkm.-

Kohlm. Systema Ascomycetum 16: 11, 1998.

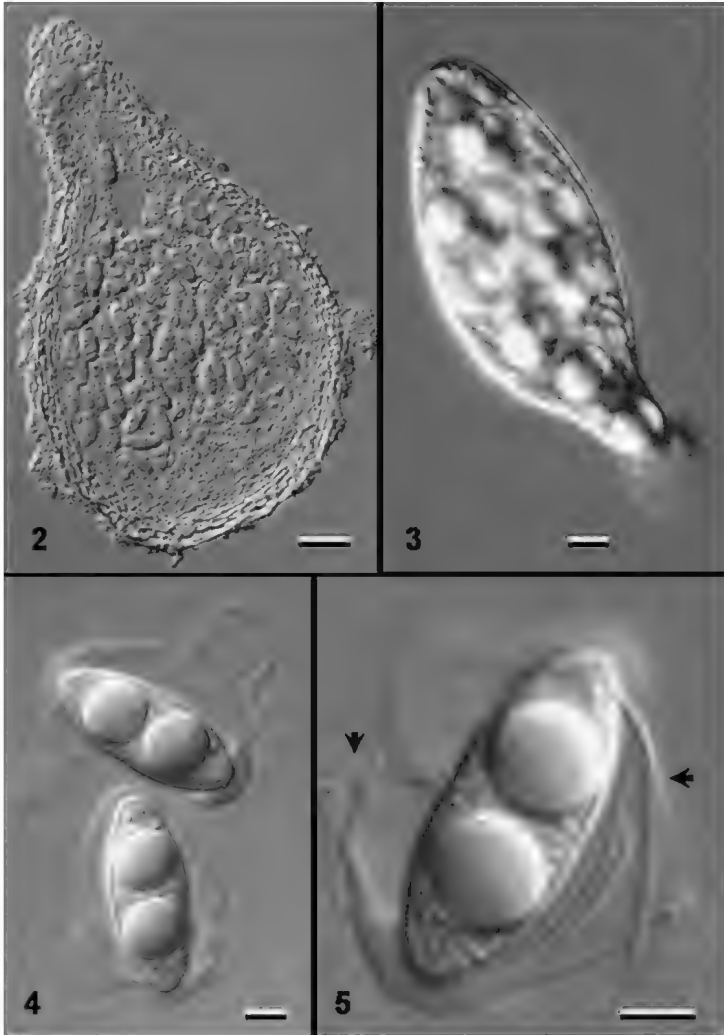
***Nohea delmarensis* (Kohlm. & Volkm.-Kohlm.) Abdel-Wahab, comb. nov.**

MYCOBANK MB 519504

FIGS 2–5

≡ *Naufragella delmarensis* Kohlm. & Volkm.-Kohlm., Systema Ascomycetum 16: 10, 1998.

Ascomata 230–260 µm high, 250–270 µm in diam, erumpent to superficial, ostiolate with short neck, periphysate, coriaceous, hyaline to cream colored,



FIGS 2-5: *Nohea delmarensis*. Differential interference contrast light micrographs. 2. Vertical section through ascoma. 3. Immature ascus. 4-5. Ascospores with elaborate two types of appendages (FIG. 5, arrowed). Bars: 2 = 50 μ m, 3-5 = 5 μ m.

solitary or gregarious (FIG. 2). Peridium 12–15 μ m thick consisting of 4–6 layers of flattened polygonal cells. Neck 50–90 μ m high, 40–48 μ m in diameter. Ascus 58–70 \times 18–27 μ m, 8-spored, thin-walled, clavate, pedunculate, deliquescent at maturity (FIG. 3). Ascospores 19–21 \times 8.5–9 μ m, one-septate, not constricted at the septum, ellipsoidal, with tapered ends. Appendages of two types: (1) polar,

gelatinous, forming a cap-like structure at the apex and lying flat against the lateral wall forming two unequal wings, wings unfurling in water to form long sticky filaments and (2) sub-polar crown of spines 15–20 µm long (FIGS 4–5).

Marinospora calyptrata (Kohlm.) A.R. Caval.

FIGS 6–10

During the course of this study, the type species of *Marinospora*, *M. calyptrata*, was recorded, described, photographed, isolated and sequenced.

Ascomata 650–850 µm high and 900–1150 µm wide, deeply immersed with long neck growing through the substrate to the surface, subglobose to fusiform or flattened globose. Asci 120–180 × 24–41 µm, 8-spored, clavate with beaked apex, deliquescing at maturity (FIG. 6). Catenophyses present. Ascospores 26–35 × 10–16 µm, one-septate, slightly constricted at the septum, ellipsoidal, hyaline, rough walled (only visible at 1000×). (FIGS 7, 9, 10), enclosed in a sheath, provided with four to five obclavate, tapering appendages. Appendages straight or curved, one at each ascospore apex and two to three around the mid-septum, 14–26 µm long, 5–8 µm wide at base; annulated along appendage length and bearing a terminal inverted cap (FIGS 7–10).

NOTE: *Marinospora longissima* was considered conspecific with *M. calyptrata* (Kohlmeyer & Kohlmeyer 1979). However, Jones & Moss (1980) separated the two species based on the presence of a mucilaginous envelope in *M. longissima* and consistently longer apical appendages. Phylogenetic analyses of LSU rDNA sequences of *M. calyptrata* and *M. longissima* confirm that they are distinct species and this is in agreement with previous study carried by Sakayaroj et al. (2004).

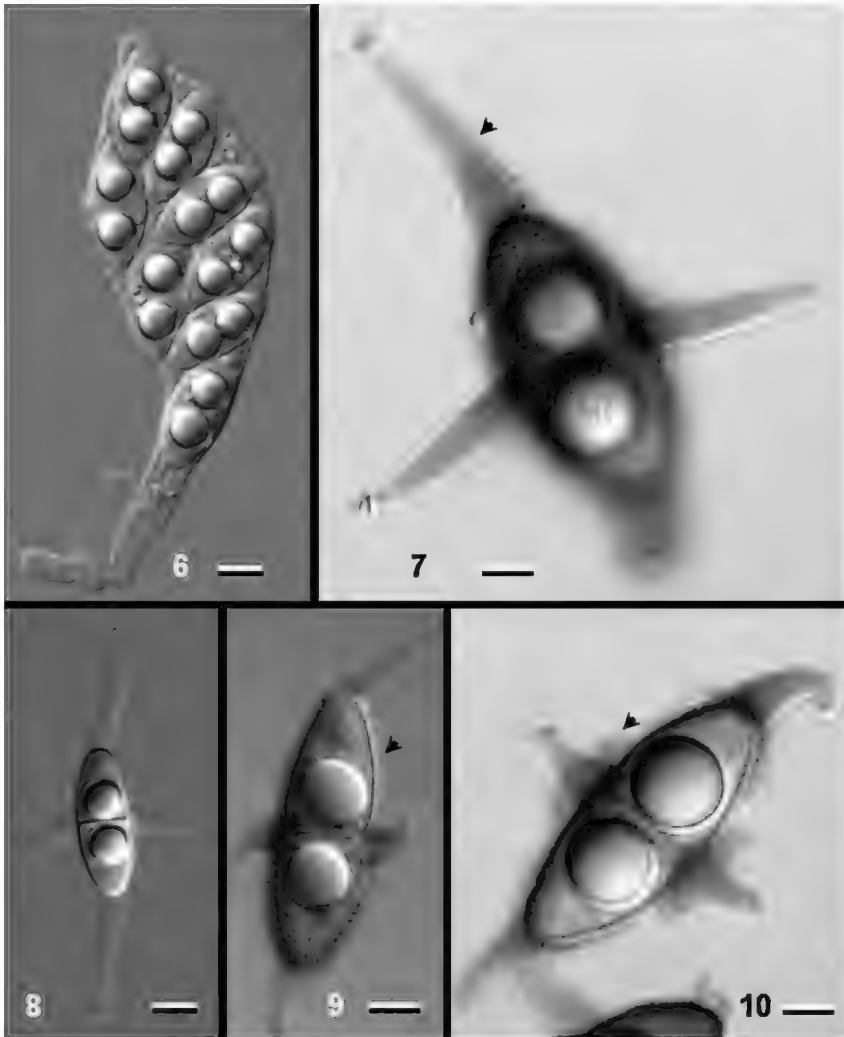
New morphological features of *M. calyptrata* observed during this study include: (1) presence of a roughened ascospore wall (FIG. 9, arrowed) surrounded by a mucilaginous layer (FIG. 10, arrowed); (2) ascospore appendages are annulated along their length (FIG. 7, arrowed). *Marinospora calyptrata* grouped consistently with *M. longissima* and *Ceriosporopsis halima*, based on molecular data (FIG. 1).

Remispora maritima Linder

FIGS 11–16

During the course of this study, the type species of *Remispora*, *R. maritima*, was isolated, photographed, and sequenced.

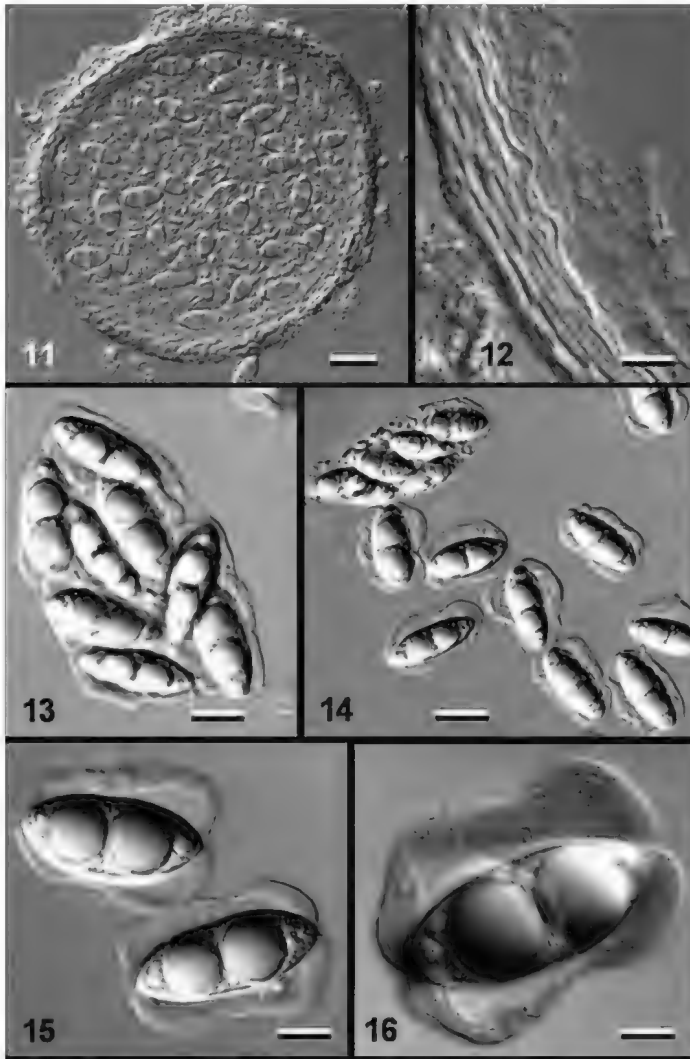
Ascomata 180–220 µm high, 250–310 µm in diam, erumpent to superficial, papillate, ostiolate, coriaceous, hyaline to cream colored (FIG. 11). Peridium 18–24 µm thick, consisting of 5–7 layers of flattened polygonal cells (FIG. 12). Neck 45–70 µm high, 60–68 µm in diam. Asci 90–120 × 26–48 µm, 8-spored, thin-walled, broadly clavate, pedunculate, deliquescing at maturity (FIG. 13). Ascospores 25–28 × 10–12 µm, one-septate, not constricted at the septum, ellipsoidal, hyaline, appendaged; at first surrounded by a gelatinous sheath



Figs 6–10: *Marinospora calyprata*. 6. Ascus. 7–10. Ascospores with annulations along the appendages (FIG. 7, arrowed), roughened wall (FIG. 9, arrowed), and gelatinous sheath (FIG. 10, arrowed). Bars: 6,8 = 10 μ m, 7,9,10 = 5 μ m.

that unfolds (FIGS 14–15), remaining attached at both ends of the ascospore; appendages later elongate and forming numerous fiber-like elements embedded in the gelatinous material (FIG. 16).

NOTE: Phylogenetic analyses of ribosomal gene sequences indicate that *Remispora* is a polyphyletic genus (Jones et al. 2009). *Remispora maritima* seems



Figs 11–16: *Remispora maritima*. 11. Section through the ascoma. 12. Section through part of the ascomata showing peridium structure. 13. Ascus. 14–16. Ascospores. Bars: 11 = 25 μm , 12 = 5 μm , 13–14 = 10 μm , 15–16 = 5 μm .

to be cosmopolitan in its distribution but occurs more frequently in temperate regions (Kohlmeyer & Kohlmeyer 1979). In the LSU analyses in this paper, *Remispora maritima* was basal to a node containing *Aniptodera chesapeakensis*, *Ascosacculus heteroguttulatus*, *Nais inornata*, and *Okeanomyces cucullatus* (FIG. 1).

Trichocladium achrasporum (Meyers & Moore) Dixon ex Shearer & J.L. Crane

Shearer & Crane (1977) discovered the connection between *Halosphaeriopsis mediosetigera* and *T. achrasporum* through single spore cultural connection. That anamorph–teleomorph connection is supported at molecular level for the first time herein. Phylogenetic analyses of LSU rDNA sequences of two isolates of *T. achrasporum* that were isolated from Japan (MF992) and Egypt (MF41) were consistently grouped with the teleomorph *H. mediosetigera* with high bootstrap support (73/96/86 for ML/MP/Bayesian pp respectively). Apparently there are several varieties of *T. achrasporum*. The type specimen has conidial dimensions 17–21(–32) × (8–)10–13 (–16) µm (Meyers & Moore 1960), while Kohlmeyer & Volkmann-Kohlmeyer (1991) reported a wider range of conidial dimensions (–15)20–34(–45) × (8–)10–24 µm. The isolates *T. achrasporum* from Egypt and Japan have similar conidial dimensions (14–32 × 8–18 µm) that agree with those of the type specimen.

Goh & Hyde (1999) monographed *Trichocladium*, accepting 18 species and describing a number of new species. They referred another 22 *Trichocladium* names to other genera, e.g. *Bactrodesmium*, *Hemispora*, and *Pithomyces*. Several *Trichocladium* species have been described from aquatic environments (Crane & Shearer 1978, Kohlmeyer & Volkmann-Kohlmeyer 1995, Hyde & Goh 1998, 1999, Goh & Hyde 1999, Hyde et al. 1999, Jones et al. 2001). *Trichocladium achrasporum* differs from the lectotype species, *T. asperum* Harz, by having sporodochioid conidiomata (Meyers & Moore 1960). Kohlmeyer & Kohlmeyer (1979), however, considered that the degree of conidiophore aggregation in *T. achrasporum* could not be considered as a sporodochium and so accepted its relocation in *Trichocladium* from the genus *Calcialna* Meyers & R.T. Moore. Currently, there are seven marine *Trichocladium* species (Jones et al. 2009).

Discussion

Ceriosporopsis halima and *Corollospora maritima* were the most frequently collected fungi during this study and these results are similar to those of previous studies that have been carried out in Japan (Tubaki 1966, Nakagiri et al. 1999). Both fungi are cosmopolitan species that have been recorded from various continents (Cribb & Cribb 1956, Kohlmeyer & Kohlmeyer 1979, Miller & Whitney 1981, Lintott & Lintott 1982, Zaniak & Jones 1984, Jones 1985, Shearer & Burgos 1987, Hyde & Jones 1989, Tan & Leong 1992, Abdel-Aziz 2010).

Halosphaeria appendiculata was frequently recorded during the present study. This species was commonly recorded from San Juan Island (Jones 1985) and from sea foam samples in Japan (Nakagiri 1989). Although cosmopolitan in its distribution, *H. appendiculata* tends to occur more frequently in temperate water (Kohlmeyer & Kohlmeyer 1979).

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